Monovalent cation effects on intermolecular purine – purine – pyrimidine triple-helix formation

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ABSTRACT

The binding of a 19-mer guanosine-rich oligodeoxyribo-nucleotide, $TG_3TG_4TG_4TG_3T$ (ODN 1), to a comple-mentary polypurine DNA target was investigated by DNase I footprinting and restriction endonuclease protection assays. Monovalent cations inhibited inter-molecular purine – purine – pyrimidine triple-helical DNA formation, with K^+ and Rb^+ being most effective, followed by NH_4+ and Na^+. Li^+ and Cs^+ had little to no effect. Similar results were observed with the G/A-rich oligonucleotide $AG_3AG_4AG_4AG_3AGCT$. Kinetic studies indicated that monovalent cations interfered with oligonucleotide – duplex DNA association but did not significantly promote triplex dissociation. The observed order of monovalent cation inhibition of triplex formation is reminiscent of their effect on tetraplex formation with G/T-rich oligonucleotides. However, using electrophoretic mobility shift assays we found that the oligonucleotide ODN 1 did not appear to form a four-stranded species under conditions promoting tetraplex formation. Taken together, our data suggest that processes other than the self-association of oligonucleotides into tetraplexes might be involved in the inhibitory effect of monovalent cations on purine – pyrimidine – purine triplex formation.

INTRODUCTION

A potentially powerful way to modulate gene expression at the level of transcription involves the disruption of specific transcription factor binding to promoter or enhancer elements by oligonucleotide-mediated triple-helix formation (1,2). Two classes of DNA triple helices, or triplexes, have been described. Both contain oligonucleotides binding in the major groove of duplex DNA through hydrogen bonding interactions with runs of purine acceptors.

In the more commonly described motif, pyrimidine oligonucleotides bind with a parallel orientation relative to the homopurine strand through Hoogsteen base pairing. Typically this involves recognition of adenosine by thymidine ($T:A-T$) and guanosine by either protonated cytidine or 5-methyl-cytidine ($C^+:G-C$) (3,4), though recognition of runs of $G-C$ base pairs

by guanosine has also been described (5). Often this requirement for cytidine protonation limits the utility of parallel motif triplexes under physiological conditions. In the second motif, purine oligonucleotides bind with an antiparallel orientation through reverse Hoogsteen base pairing (6,7). Base pairing in this motif includes guanosine binding to guanosine ($G:G-C$) and either adenosine or thymidine binding to adenosine ($A:A-T$ or $T:A-T$). These purine – purine – pyrimidine ($Pu-Pu-Py$) triplexes have been shown to exist at physiological pH, Mg^{2+}, and polyvalent cation concentrations, thus making the purine motif the preferred approach to antigene therapy (8–12).

While investigating transcription inhibition in vitro through triplex formation, we discovered that purine motif triplexes did not efficiently form under standard in vitro transcription conditions. The most significant difference between triplex-formation and in vitro transcription conditions was the presence of moderate (60 mM) KCl concentrations in the latter. KCl is known to promote the formation of four-stranded, or tetraplex, species with G/T-rich oligonucleotides (13,14). Since the environmental parameters underlying $Pu-Pu-Py$ triplex formation have not been as well described as for $Py-Pu-Py$ triplexes, we undertook a systematic study of monovalent cation effects on purine-motif triplex formation. DNase I footprinting and restriction endonuclease protection assays were used to investigate $Pu-Pu-Py$ triplex formation and stability; electro-phoretic mobility shift assays were used to investigate changes in oligonucleotide structure resulting from monovalent cations. Our data suggest that moderate concentrations of several monovalent cations interfere with the association of G-rich oligonucleotides with duplex DNA, by a mechanism that does not seem to involve tetraplex formation.

MATERIALS AND METHODS

Oligodeoxyribonucleotides and DNA probes

Guanosine-rich oligodeoxyribonucleotides (ODN) used in this study included: (ODN 1) 5'-$TG_3TG_4TG_4TG_3T$-3', (ODN 2) 5'-$AG_3AG_4AG_4AG_3AGCT$-3', and (ODN 3) 5'-TTCTT-$CTTG_4TG_3T$-3'. All were synthesized by phosphoramidite chemistry on an Applied Biosystems DNA synthesizer and purified by n-butanol precipitation (15). Concentrations were

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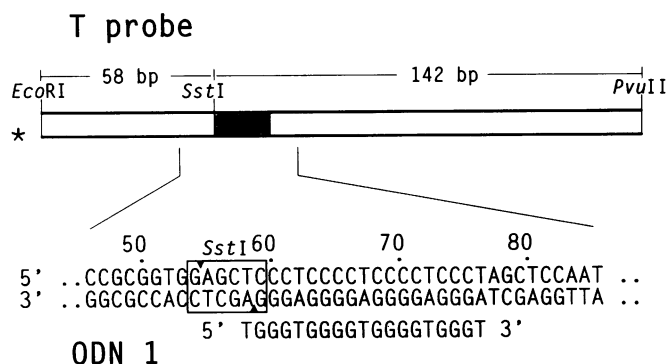
T probe

Figure 1. Structures and sequences of nucleic acids used for analyzing purine–purine–pyrimidine triplex formation. (Top) Schematic representation of the duplex DNA probe (Triplex forming or T probe) containing a homopurine–homopyrimidine cassette (shaded area). Important restriction endonuclease cleavage sites and DNA fragment lengths are indicated. Site of single, 3'-^{32}P end label is indicated by an asterisk. (Bottom) Nucleotide sequences of the homopurine–homopyrimidine cassette region and oligodeoxyribonucleotide ODN 1. Numbers above indicate the distance from the labeled 3' end. The box indicates the overlapping *Sst*I cleavage site used in the REPA assay. ODN 1 is shown adjacent to the homopurine strand with a location and orientation expected for an antiparallel-motif triplex. The control probe (C probe) used in REPA assays is identical to probe T except for the deletion of the homopurine-homopyrimidine cassette (base pairs 59–76).

determined spectrophotometrically, using an average nucleotide molar extinction coefficient at 260 nm of $3.3 \times 10^5 \, M^{-1} cm^{-1}$. Duplex DNA probes used in endonuclease protection assays consisted of the 182 bp *Eco*RI–*Pvu*II fragment from pBluescript II SK, either alone (control) or containing a 19 bp polypurine–polypyrimidine cassette cloned into the *Sac*I site (triplex-forming). Both were singly 3' end-labeled at the *Eco*RI site by Klenow end-filling and purified by NA-45 membrane (Schleicher & Schuell) following manufacturer's instructions. The structure of these probes, together with the sequence of the triplex-forming cassette, is shown schematically in Fig. 1.

DNase I footprinting and restriction endonuclease protection assays

To effect triplex formation, we incubated 50 nM duplex DNA probe and 1 μM oligonucleotide for 60 min at 30°C in a 15 μl volume containing 40 mM HEPES (pH 8.2), 12 mM MgCl$_2$ and 5 mM dithiothreitol. Additional alkali metal chloride salts were also present during this incubation, as indicated in the figure legends. For DNase I footprinting, 2 ng DNase I was added to each sample. Cleavage was allowed to proceed for 30 sec at room temperature, and the reaction was terminated by the addition of 3 μl stop buffer (0.6 M ammonium acetate, 0.1 M EDTA, 0.2 mg/ml tRNA). Adenine-specific chemical cleavage reactions were used as markers (16). For restriction endonuclease protection assays (REPA), 10 units of *Sst*I were added to each sample, and cleavage was allowed to proceed for 5 min at 30°C. For both DNase I footprinting and REPA, samples were purified by phenol/chloroform extraction and ethanol precipitation, dried briefly, resuspended in 95% formamide, and heated at 95°C for 4 min before being loaded onto a denaturing 8% polyacrylamide gel. Electrophoretically separated DNA cleavage products were visualized by autoradiography and quantitated by direct beta scanning. Determination of the extent of triplex formation by REPA followed normalization of *Sst*I cleavage efficiency with a control DNA probe present in each reaction mixture.
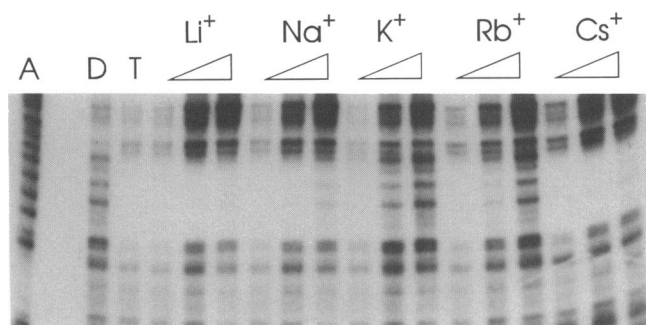
Figure 2. DNase I cleavage analysis of Pu–Pu–Py triplex formation in the presence of various alkali metal chlorides. Reactions containing 1 μM ODN 1, 12 mM MgCl$_2$, and 40 mM HEPES (pH 8.2) and 50 nM probe T were incubated for 60 min at 30°C before DNase I cleavage. Alkali metal cations, when included, were present during the entire incubation period at concentrations of 1, 10, and 100 mM, as indicated by the increasing slope of the right triangles above each set of three lanes. Lane A, adenine-specific chemical cleavage reaction. Lane D, DNase I control lane without ODN 1. Lane T, control with ODN 1 and no alkali metal chloride. The bar at right indicates the region protected against DNase I cleavage by ODN 1 binding.

Kinetic analyses

Triplex association reaction mixtures were assembled as above, except that the times of incubation ranged from 0 to 90 min before analysis by REPA. For triplex dissociation studies, triplexes were first assembled as described. Unbound oligonucleotides were then separated from triplexes by ultrafiltration (Millipore 30,000 NMWL cellulose). The retained triplexes were resuspended in 150 μl of a buffer containing 40 mM HEPES (pH 8.2) with or without 100 mM KCl as indicated. Samples were incubated at 30°C for various times (0–10 h) before analysis by REPA.

Tetraplex formation and electrophoretic mobility shift analysis

Four-stranded DNA species were assembled essentially as described (17). Briefly, 5' end-labeled oligonucleotides at a concentration of 100 μM in water were denatured at 95°C for 1 min and then chilled on ice. Aliquots were removed and diluted with an equal volume of either 2× TE (40 mM Tris (pH 7.9) and 0.4 mM EDTA), 2× TE and 2 M KCl, or 2× triplex-forming buffer (see above). These samples were incubated at room temperature for 1 h, then diluted five-fold with ice-cold loading buffer (TE, 10 mM KCl, 20% glycerol, bromophenol blue, and xylene cyanol loading dyes). The resulting DNA species were resolved by electrophoresis at 100 V on a nondenaturing 12% polyacrylamide gel containing 50 mM Tris–borate, 0.5 mM EDTA, and 10 mM KCl and visualized by autoradiography.

RESULTS

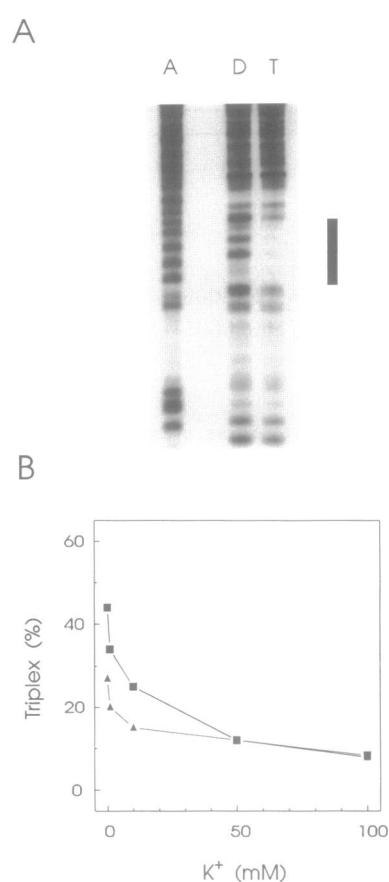### Monovalent cation effects on purine–purine–pyrimidine triplex formation

While Na$^+$ has been shown to affect Py–Pu–Py triplexes by increasing their rate of dissociation (18), the effects of monovalent cations on Pu–Pu–Py triplex formation have not been as well characterized. For these studies we chose the purine-rich oligodeoxyribonucleotide TG$_3$TG$_4$TG$_4$TG$_3$T (**ODN 1**) as our model compound, given its strong, sequence-specific binding to a G-rich homopurine, duplex DNA target (6,19). ODN 1 and DNA were incubated under conditions favoring triplex formation (12 mM Mg^{2+} buffered to pH 8.2) (6,19). Triplex formation
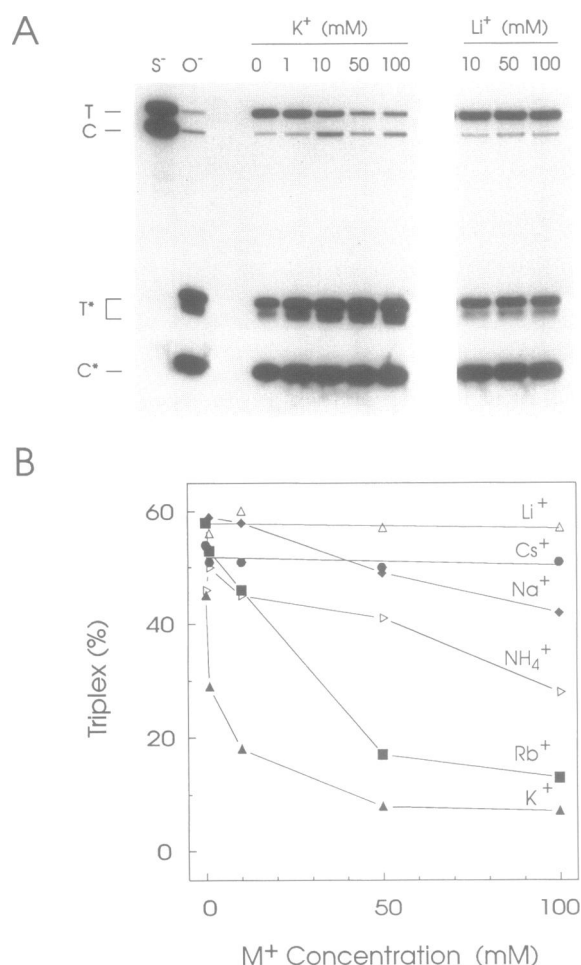
Figure 3. Formation of triplexes in the presence of alkali metal cations. Triplex formation in the presence of different concentrations of various monovalent cations was monitored using a restriction endonuclease protection assay. (**A**) Autoradiogram of a representative experiment using K$^+$ and Li$^+$. Reaction conditions were identical to those used in DNase I protection except that the restriction endonuclease SstI was used instead. Lanes S$^-$ and O$^-$ correspond to control reactions carried out in the absence of SstI and ODN 1, respectively. Labeled DNA species include uncut triplex-forming (T) and control (C) probes and the restriction fragments resulting from SstI cut T and C probes (T* and C*, respectively). (**B**) Graph of triplex formation as a function of monovalent cation concentration. Triplex formation was determined by the percentage of labeled DNA rendered resistant to SstI cleavage by interaction with ODN 1 after normalization of SstI cleavage efficiency for the internal control probe C.

Figure 4. Monovalent cations inhibit triplex formation by a G/A-rich oligonucleotide. (**A**) DNase I footprint of ODN 2 (5' AG$_3$AG$_4$AG$_4$AG$_3$AGCT 3') bound to probe T. Reaction conditions were as described in Fig. 2 except that 5 μM ODN 2 was present. (**B**) SstI cleavage protection assay for ODN 2 binding to probe T in the presence of KCl. (Solid squares) 5 μM ODN 2; (solid triangles) 1 μM ODN 2.

was investigated by DNase I cleavage protection (7). As shown in Fig. 2., oligonucleotide-dependent DNase I cleavage inhibition covering the GA strand of the polypurine—polypyrimidine cassette was observed, indicating complete formation of triple-helical DNA on all duplex DNA targets under these conditions. A similar protection pattern was also observed on the CT strand (data not shown). Addition of certain alkali metal cations during incubation had a marked effect on the extent but not the overall pattern of DNase I cleavage protection. While Li$^+$ and Cs$^+$ had little effect on triplex formation, K$^+$ and Rb$^+$ inhibited this process significantly, with half-maximal inhibition occurring at approximately 10 and 100 mM concentrations, respectively.

While DNase I cleavage protection allows the visualization of triplex formation, this method is not readily amenable to quantitation, especially under conditions of incomplete triplex

formation. In order to more accurately determine monovalent cation effects on Pu—Pu—Py triplexes, we developed a restriction endonuclease protection assay (**REPA**). In this assay, triplex formation was monitored by its interference with the cleavage of a restriction endonuclease directed to an adjacent site (Fig. 1). Triplex formation was quantitated by comparing the amount of uncut with cut probe following electrophoretic separation. An example of this assay is shown in Fig. 3(A). In our REPAs we included as an internal control a second DNA probe of a different length that contains an identical SstI restriction endonuclease cleavage site without the adjacent triplex-forming cassette. This control fragment was used to verify the completeness of SstI digestion under the variety of high ionic strengths used. Quantitation of our REPAs is shown graphically in Fig. 3(B). We found that many monovalent cations interfered with triplex formation in a concentration dependent manner, the order of effectiveness being K$^+$ > Rb$^+$ > NH$_4$+ > Na$^+$ > Li$^+$ or Cs$^+$. While 50% inhibition of triplex formation occurred with less than 10 mM K$^+$, most other cations required concentrations greater than 100 mM to show similar effects.

To test the universiality of triplex inhibition by monovalent cations, we investigated a second oligonucleotide, AG$_3$AG$_4$A-G$_4$AG$_3$AGCT (**ODN 2**), capable of forming Pu—Pu—Py
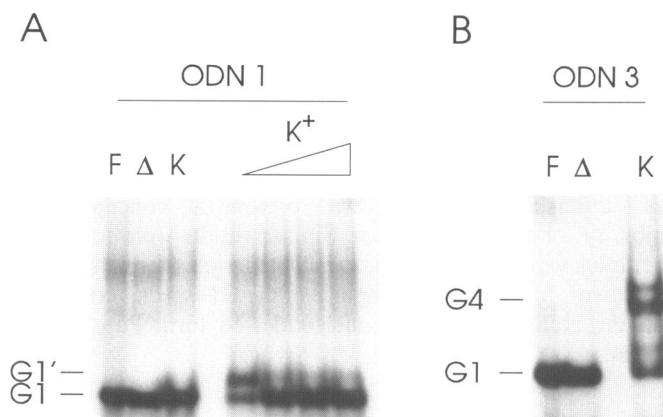
A

B
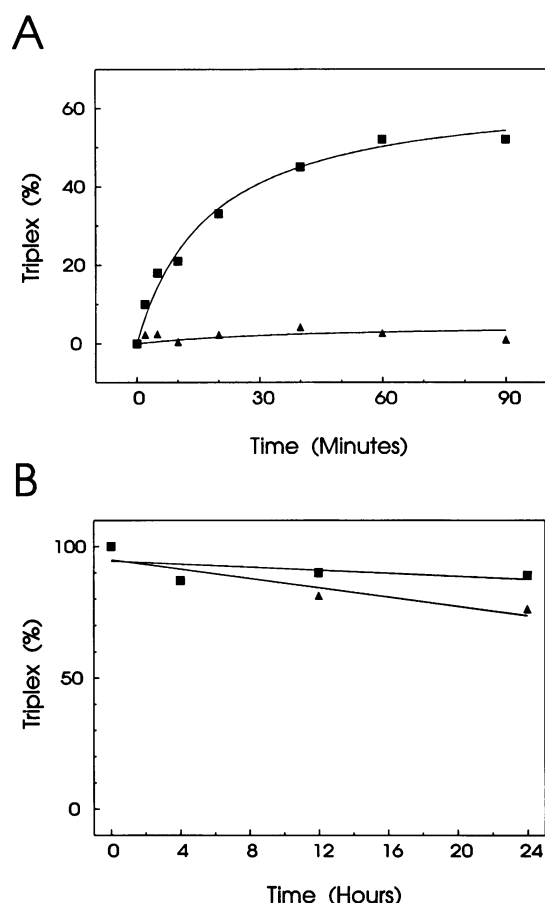
Figure 5. Effects of monovalent cations on the formation and dissociation of Pu−Pu−Py triplexes. Reaction conditions were those described in the Materials and Methods section. Solid squares and triangles refer to reactions containing 0 and 100 mM KCl, respectively. (A) Time course of ODN 1 binding to the duplex DNA probe T. (B) Time course of dissociation for the ODN 1-probe T triplex.

A

ODN 1

B

ODN 3

Figure 6. Electrophoretic mobility shift analysis of G/T-rich oligonucleotides. In each reaction, 50 mm ODN was present during the 60 min incubation at room temperature. (A) Electrophoretic mobility of ODN 1 species. Lane F, formamide-denatured ODN. Lane D, heat-treated ODN. Lane K, ODN treated with 1 M KCl. Lanes K^+, ODN 1 incubated under triplex-forming conditions (40 mM HEPES, pH 8.2 and 12 mM $MgCl_2$) with the addition of increasing concentrations of KCl (0, 1, 10, and 100 mM). G1 indicates the migration of the putative ODN 1 monomer, while G1′ points to a different Mg^{2+}-dependent form of ODN 1. (B) Electrophoretic mobility shift assay of ODN 3 species. G1 and G4 refer to the putative monomer and tetraplex species, respectively.

triplexes. Such triplexes can be formed under conditions similar to those used for G/T-rich oligonucleotides, albeit less efficiently (6). As shown in Fig. 4(A), we found a DNase I footprint for ODN 2 that was similar to that for ODN 1, although a five-fold greater concentration of oligonucleotide was required. REPAs using ODN 2 at two different concentrations (Fig. 4B) indicated that triplex formation with this oligonucleotide was also sensitive to increasing K^+ concentrations. Comparison of Figs 3(B) and 4(B) indicates that G/T- and G/A-rich oligonucleotides responded almost identically to K^+.

Kinetic analysis of K^+ effects on triplexes

The inhibition of triplex formation by monovalent cations could reflect either an inhibition of ODN−DNA association or a destabilization of preformed triple helices. For Py−Pu−Py triplexes, it was reported that high Na^+ concentrations (0.1−1 M) tend to decrease slightly the association rates while significantly increasing the dissociation rates (18). However, it has also been reported that the association rate for Py−Pu−Py triplexes decreased markedly upon decreasing Na^+ concentration (from 300 to 20 mM) while the dissociation rate was not significantly affected (20).

We used the restriction endonuclease protection assay to determine the effects of K^+ on the kinetics of Pu−Pu−Py triplex formation and dissociation. These experiments are shown in Fig. 5. At micromolar concentrations of ODN 1, Pu−Pu−Py triplex formation in the absence of K^+ exhibited saturation kinetics characterized by 50% maximal binding being completed after 12 min. However, in the presence of 100 mM K^+, triplex formation occurred far more slowly, with less than 10% accumulation observed after 60 min. With regards to the stability of Pu−Pu−Py triplexes, we found a slightly higher rate of dissociation in the presence of 100 mM K^+ than in its absence (Fig. 5B). However, in both cases the half-lives of these triplexes was on the order of days. Thus unlike Py−Pu−Py triplexes, steps leading to Pu−Pu−Py triplex formation tend to be the most sensitive to inhibition by monovalent cations such as K^+.

K^+ and Mg^{2+} effects on oligodeoxyribonucleotide self-association

Two possible explanations exist for the observed monovalent cation effect on Pu−Pu−Py triplex formation. Potassium ions might directly inhibit oligonucleotide-duplex DNA interaction. Alternatively, K^+ could stabilize an oligonucleotide species that is refractory to triplex formation. It has been shown that G/T-rich oligonucleotides self-associate in the presence of certain monovalent cations to form four-stranded, or tetraplex, species (13,14). In fact, our observed sensitivity to monovalent cations, $K^+ > Rb^+ > Na^+ > Li^+$ or Cs^+, corresponds exactly to that observed for stabilizing certain tetraplex DNAs (14). In order to determine whether stabilization of tetraplexes was responsible for the inhibition of triplex formation by monovalent cations, we investigated the state of our oligonucleotides under triplex-forming conditions by an electrophoretic mobility shift assay. As a positive control, ODNs were submitted to conditions that promote the formation of higher order structures such as tetraplexes, i.e. elevated temperatures and molar concentrations

of K^+ (17). As shown in Fig. 6, ODN 1 did not change its electrophoretic mobility under conditions that promoted tetraplex formation for the oligonucleotide TTCTTCTTG$_4$TG$_3$T (**ODN 3**). However, under conditions that allowed formation of Pu−Pu−Py triplexes, ODN 1 exhibited a slightly slower mobility (G1′) than denatured ODN 1. Appearance of this G1′ species was diminished when K^+ was present at concentrations inhibitory for triplex formation. Similar results were found for other monovalent cations that also inhibited triplex formation (data not shown). In all cases, appearance of the G1′ species correlated with the ability of the oligonucleotides to form Pu−Pu−Py triplexes.

DISCUSSION

Several monovalent cations were found to interfere with Pu−Pu−Py triplex formation even at concentrations less than 100 mM. Potassium ions were the most effective inhibitors, with other cations being less inhibitory as their ionic radii differed further from that of K^+. This phenomenon explains our prior inability to affect specific RNA polymerase II-dependent transcription by antiparallel motif triplexes, given the presence of 60 mM KCl in standard *in vitro* transcription assays (21). Our results show that loss of triplex formation in the presence of certain monovalent cations occurred at the level of oligo-nucleotide−duplex DNA association; the dissociation rate of Pu−Pu−Py triplexes was not appreciably affected by 100 mM KCl.

These findings have important practical applications. Since they are very stable, it should be possible to assemble triplexes in the absence of KCl and have them survive through the course of an *in vitro* transcription assay. Alternatively, nuclear extracts for use in transcription studies with triplexes could be prepared with an alkali chloride salt other than KCl. Such modifications to the standard protocol should make it possible to investigate triplex-mediated eukaryotic transcription inhibition *in vitro*. However, transcription inhibition by Pu−Pu−Py triplexes *in vivo* might not be feasible unless cellular factors exist that abrogate the effects of physiological KCl concentrations. While several laboratories have reported that G/T-rich oligonucleotides inhibited transcription *in vivo*, none have directly demonstrated triplex formation *in vivo* (9−12). A possible explanation for these conflicting results would be that the single-stranded G/T oligonucleotides by themselves inhibit gene-specific transcription by competing for certain DNA-binding *trans*-acting factors. One such example is the HeLa *Pur* protein, which binds tightly to single-stranded oligonucleotides whose sequences are found within the human c-*myc* promoter (22,23).

How do monovalent cations inhibit triplex formation? Monovalent cations could stabilize an oligonucleotide species that is refractory to association with duplex DNA. One such candidate species is tetraplex, or G-4 DNA (14,17). In G-4 DNA, the four DNA strands have a parallel orientation and interact with one another through Hoogsteen-bonded guanine quartets. These tetraplexes are stabilized by the presence of monovalent cations, especially those that we found were most effective in preventing triplex formation. While we did not observe G-4 DNA formation with ODN 1, either through changes in its electrophoretic mobility (Fig. 6A) or in its pattern of reactivity to the alkylating agent dimethylsulfate (A.-J.Cheng and M.W.Van Dyke, unpublished observations), it cannot be completely excluded that other self-associated species, for example, a dimer of hairpin-

shaped strands (G′2-DNA), might be involved (13,14,24). However, given the observed identical electrophoretic mobilities of both formamide-denatured and KCl-treated oligonucleotides, such a species would have to form under the somewhat suboptimal conditions present in both the loading buffer and during gel electrophoresis (i.e., 10 mM K^+, 10 μM ODN). Milligan *et al.* have described ODNs containing 7-deaza-2′-deoxyguanosine that are capable of antiparallel motif triplex formation yet are equally inhibited by 140 mM K^+ as their corresponding G/T-rich oligonucleotide counterparts (25). Given that these ODNs are unable to self-associate through Hoogsteen base pairing, this would argue that the inhibition of purine−purine−pyrimidine triplex formation by certain monovalent cations does not result from a decrease in the available ODN concentration following formation of such self-associated oligonucleotide species.

Alternatively, monovalent cations could directly interfere with oligonucleotide-duplex DNA association through a competition with divalent cations required for triplex formation. Such has been reported for pyrimidine−purine−pyrimidine triplexes at high NaCl concentrations (18). Displacement of divalent cations would seem rather unlikely under our reaction conditions, given the relative concentrations of Mg^{2+} (12 mM) and K^+ (1 mM) when triplex inhibition first occurs.

A third possibility would be that oligonucleotides assume a particular conformation prior to their association into triplexes, and that this isomerization can be inhibited by certain monovalent cations. In antiparallel motif triplexes, all nucleic acid bases are thought to reside with *anti* conformations with respect to their glycosidic bonds (6). Under the proper conditions, such oligonucleotides might be expected to interact with one another through Hoogsteen base pairing similar to that found in Pu−Pu−Py triplexes. Our G1′ species could well correspond to such a G/T-oligonucleotide dimer. Such a species might be an intermediate along the reaction pathway leading to triplex formation. Thus destabilization of this intermediate by monovalent cations would result in a decrease in the kinetics of triplex formation. Alternatively, if the association between certain monovalent cations and G/T-rich oligonucleotides was favored thermodynamically, this stabilization of the monomer DNA species would tend to shift the equilibrium away from triplex formation. Either possibility is consistent with our data.

ACKNOWLEDGEMENTS

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